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Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*

(autoinducers/quorum sensing/gene regulation)

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ABSTRACT **_Pseudomonas aeruginosa_ produces a spectrum of exoproducts many of which have been implicated in the pathogenesis of human infection. Expression of some of these factors requires cell–cell communication involving the interaction of a small diffusible molecule, an "autoinducer," with a positive transcriptional activator. In _P. aeruginosa_ PAO1, LasI directs the synthesis of the autoinducer _N_-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), which activates the positive transcriptional activator, LasR. Recently, we have discovered a second signaling molecule-based modulon in PAO1, termed _vsm_, which contains the genes _vsmR_ and _vsmI_. Using HPLC, mass spectrometry, and NMR spectroscopy we now establish that in _Escherichia coli_, VsmI directs the synthesis of _N_-butanoyl-L-homoserine lactone (BHL) and _N_-hexanoyl-L-homoserine lactone (HHL). These compounds are present in the spent culture supernatants of _P. aeruginosa_ in a molar ratio of ≈15:1 and their structures were unequivocally confirmed by chemical synthesis. Addition of either BHL or HHL to PAN067, a pleiotropic _P. aeruginosa_ mutant unable to synthesize either of these autoinducers, restored elastase, chitinase, and cyanide production. In _E. coli_ carrying a _vsmR/vsmI'::lux_ transcriptional fusion, BHL and HHL activated VsmR to a similar extent. Analogues of these _N_-acyl-L-homoserine lactones in which the N-acyl side chain has been extended and/or oxidized at the C-3 position exhibit substantially lower activity (e.g., OdDHL) or no activity (e.g., dDHL) in this _lux_ reporter assay. These data indicate that multiple families of quorum sensing modulons interactively regulate gene expression in _P. aeruginosa_.**

The recognition that *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; Fig. 1), previously termed the *lux* autoinducer (AI) (1), regulates both secondary metabolism (2, 3) and virulence in the plant pathogen *Erwinia carotovora* (4, 5) has been followed by a surge of interest in the role of *N*-acyl-L-homoserine lactones (AHLs) in bacterial gene expression (6–8). Diverse Gram-negative bacteria are now known to synthesize AHLs which form part of a cell–cell communication system that facilitates the induction of genetic regulons only when a significant cell population density has been attained (2, 6–8). This cell density dependency reflects the accumulation of the signal molecule to a critical threshold concentration (1, 6–8) and is termed quorum sensing (6). Quorum sensing systems depend upon the interaction of an AI, the synthesis of which is directed by a LuxI homologue, with a positive transcriptional activator encoded by a *luxR* homologue. *luxI* and *luxR* were originally described in *Photobacterium* (*Vibrio*) *fischeri*, where OHHL and LuxR interact to regulate the induction of bioluminescence (1). Since then, homologues of LuxI and LuxR have been identified in *Er. carotovora* (5, 9, 10), *Agrobacterium tumefaciens* (11–13), *Pseudomonas aeruginosa* (14–17), *Pseudomonas aureofaciens* (18), *Yersinia enterocolitica* (19), *Enterobacter agglomerans* (9), and *Rhizobium leguminosarum* (20).

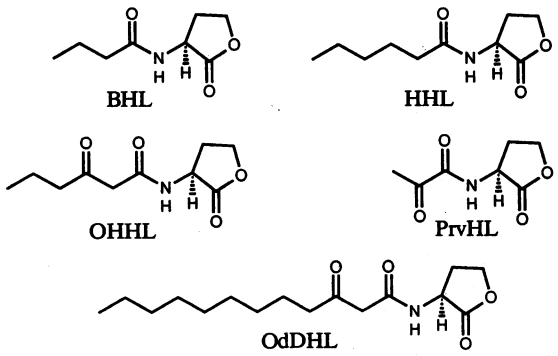

FIG. 1. Structures of some AHLs used in this work.

The first AHL identified in culture supernatants of *P. aeruginosa* PAO1 was OHHL (2). Later the *luxI* homologue *lasI* was identified in PAO1 and shown to direct the synthesis primarily of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL; Fig. 1) (15, 21). However, supernatants from PAO1 and from *Escherichia coli* transformed with *lasI* also contained a compound which was coeluted with the same HPLC retention time as OHHL (21).

P. aeruginosa PAN067 is a pleiotropic mutant derived from PAO1 which lacks the ability to produce elastase, alkaline protease, pyocyanin, hemolysin, and cyanide and is also defective in AHL production (4, 17). Elastase synthesis in PAN067 can be restored to some extent by OHHL but not by OdDHL (4, 17). From a cosmid library prepared from PAO1 chromosomal DNA, we identified a genetic locus capable of complementing PAN067 and restoring exoproduct

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Abbreviations: AI, autoinducer; AHL, *N*-acyl-L-homoserine lactone; BHL, *N*-butanoyl-HL; HHL, *N*-hexanoyl-HL; OHL, *N*-octanoyl-HL; dDHL, *N*-dodecanoyl-HL; OBHL, *N*-(3-oxobutanoyl)-HL; OHHL, *N*-(3-oxohexanoyl)-HL; OOHL, *N*-(3-oxooctanoyl)-HL; OdDHL, *N*-(3-oxododecanoyl)-HL; PrvHL, *N*-pyruvoyl-HL; EI, electron impact; FAB, fast atom bombardment.

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synthesis (17). This locus encodes homologues of LuxR and LuxI which are distinct from LasR and LasI and are termed VsmR and VsmI, respectively, to indicate their role in regulating both virulence determinants and secondary metabolites (17). These genes have also been described as *rhlR* and *rhlI* (16, 17).

The identification of VsmI implies the existence of other AHL signaling molecule(s) distinct from OdDHL. Here we show that VsmI directs the synthesis of *N*-butanoyl-L-homoserine lactone (BHL; Fig. 1) and *N*-hexanoyl-L-homoserine lactone (HHL; Fig. 1) and that these AHLs can induce exoproduct synthesis in PAN067. Using various synthetic AHLs in conjunction with a *vsmR/vsmI'::lux* transcriptional fusion, we evaluate the structural requirements necessary for activation of VsmR.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The *P. aeruginosa* strains used were PAO1 and the PAO1 mutant PAN067 (4). *E. coli* JM109 was transformed with pMW47.1 (*vsmRI*; ref. 17), pMW473.1 (*vsmI*; ref. 17), or the reporter plasmid pSB406 (see below). Bacteria were grown with shaking in Luria–Bertani (LB) medium at 30°C except for purification of AHLs, for which *E. coli* JM109(pMW47.1) was grown with shaking at 30°C in M9 medium (22) plus 0.01% thiamine and proline; *P. aeruginosa* strains were grown in M9 medium plus 10 mM succinic acid. Where required for plasmid maintenance, ampicillin (50 μg/ml) and carbenicillin (500 μg/ml) were used for *E. coli* and *P. aeruginosa*, respectively.

AI Assays. AIs were detected with a *Chromobacterium violaceum* mutant (CV026) which responds to a range of AHLs (with N-acyl side chains of four to eight carbons irrespective of the oxidation state at C-3) by inducing the synthesis of the purple pigment violacein (17, 23). Spent culture supernatants or extracts were added to wells cut in nutrient agar plates seeded with CV026, incubated at 30°C overnight, and then examined for the presence of violacein.

To determine the structure–activity profile for AHLs capable of activating VsmR, synthetic AIs at 0–15.6 μM were added to LB containing *E. coli* JM109(pSB406). In this bioluminescent *E. coli*-based reporter system, the *vsmR* gene and the *vsmI* promoter region have been coupled to the *lux* structural operon (*luxCDABE*) of *Photorhabdus luminescens* (to be described in detail elsewhere). After 2 hr of incubation, bioluminescence (as relative light units) was recorded with a Berthold (Nashua, NH) LB980 imaging system.

Purification and Identification of AHLs. Supernatants from stationary-phase cultures of PAO1 or *E. coli* JM109(pMW47.1) were extracted with dichloromethane. AHLs were partially purified by chromatography on silica gel columns with dichloromethane/methanol (98:2, vol/vol) as eluant, followed by reverse-phase semipreparative HPLC (Kromasil KR100-5C8 column, 250 mm × 8 mm; Hichrom, Reading, U.K.) with an isocratic mobile phase of 70% (vol/vol) acetonitrile in water at a flow rate of 2 ml/min and monitored at 210 nm. Fractions showing activity in the CV026 bioassay were pooled and rechromatographed with 60% acetonitrile and the procedure was repeated with 35% acetonitrile in water. Active fractions were analyzed by MS and ^{1}H NMR [Bruker (Billerica, MA) AM400 spectrometer operating at 400 MHz]. Mass spectra were obtained on a VG 70-SEQ instrument of EBqQ geometry (Fisons Instruments, VG Analytical, Manchester, U.K.). Samples were ionized by either electron impact (EI) or positive-ion fast atom bombarbment (FAB). The molecular ion (M + H) peaks recorded by FAB-MS were further analyzed by tandem MS (MS–MS) and shown to have MS–MS spectra identical to those of the respective authentic synthetic materials.

Synthesis of AHLs. The general synthetic method of Chhabra *et al.* (24) was used to produce BHL, HHL, *N*-octanoyl-L-homoserine lactone (OHL), *N*-dodecanoyl-L-homoserine lactone (dDHL), and their 3-oxo derivatives, OBHL, OHHL, OOHL, and OdDHL. In addition, *N*-pyruvoyl-L-homoserine lactone (PrvHL; Fig. 1) was synthesized. Each compound was purified to homogeneity by semipreparative HPLC and its structure was confirmed by MS and ^{1}H NMR spectroscopy.

Exoproduct Assays. BHL or HHL (0, 25, or 100 μM) was added to the growth medium of PAN067, and restoration of elastase synthesis was evaluated qualitatively by Western blotting (17) and quantitatively by the elastin–Congo red assay (14). Chitinase activity (25) and cyanide production (26) were assayed as described.

RESULTS

Purification of AIs. *P. aeruginosa* PAN067 is defective in the production of exoproducts and AI(s) (4, 17) but can be

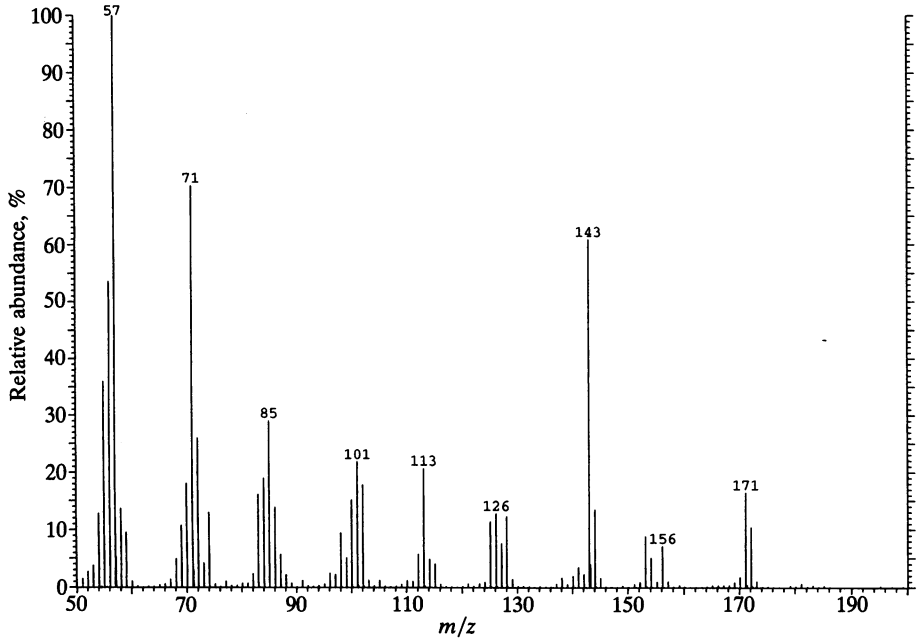

FIG. 2. EI-MS of AI-1 purified from spent culture supernatants of *E. coli* JM109(pMW47.1). The molecular ion at *m/z* 171 is tentatively assignable to either BHL or PrvHL.

complemented with a *P. aeruginosa* PAO1 DNA fragment containing *vsmR* and *vsmI*, which encode homologues of LasR and LasI (17). PAO1 thus possesses at least two LuxI homologues, and since LasI is responsible for OdDHL synthesis, VsmI is likely to direct the synthesis of a novel AI. To explore this possibility, we used a bioassay for AHLs that depends on the induction of violacein in the *C. violaceum* mutant CV026 (17, 23). Although PAO1 culture supernatant induced violacein production, neither synthetic OdDHL nor PAN067 supernatant could induce pigmentation (ref. 17; data not shown). This observation prompted us to exploit CV026 in the purification of AHLs from supernatants of PAO1 and of *E. coli* JM109 carrying pMW473.1 or pMW47.1 (17). Since the putative AI(s) was produced in greater amounts by *E. coli* JM109(pMV47.1), dichloromethane extracts of its spent culture supernatants were subjected to silica gel chromatography followed by HPLC. Active fractions were located in two single peaks (AI-1 and AI-2, respectively) with retention times in 35% acetonitrile of 5.1 and 10.1 min, respectively. Two compounds with these retention times were also obtained from *P. aeruginosa* PAO1 supernatants but were absent from PAN067 supernatants.

In addition to AI-1 and AI-2, supernatants of PAO1 contained OHHL, which has a retention time in 60% acetonitrile of 4.4 min and can activate violacein synthesis in CV026. By use of a synthetic standard, OdDHL was also provisionally identified in PAO1 supernatants, with a retention time of 14.2 min in 60% acetonitrile. OdDHL was also present in culture supernatants of PAN067 but absent from those of *E. coli* JM109(pMW47.1).

Structures of the AHLs Synthesized via VsmI. AI-1 and AI-2 isolated from *E. coli* JM109(pMW47.1) were analyzed by high-resolution MS and ^{1}H NMR spectroscopy. AI-1 displayed a molecular ion peak at m/z 172 (M + 1) in FAB-MS and an m/z 171 with major fragments at 156, 143, 126, 113, 101, 85, 71, and 57 in EI-MS (Fig. 2). These data were tentatively attributable to either BHL or PrvHL. Both compounds were synthesized and were found to share the same HPLC retention time. However, PrvHL was unable to induce violacein synthesis in CV026, indicating that the natural compound was probably BHL. The structure of AI-1 was unequivocally confirmed as BHL by ^{1}H NMR spectroscopy (Fig. 3).

AI-2, which was present in small amounts relative to AI-1, showed a very weak signal at m/z 200 (M + 1) in FAB-MS, suggesting that this compound may be HHL. The L isomer of HHL was synthesized and was eluted with the same HPLC retention time as AI-2. HHL also activated CV026, and final confirmation of the assigned structure was obtained from the MS–MS data on the m/z 200 peak, which were identical in all respects to the MS–MS data obtained with synthetic HHL (Fig. 4).

Compounds with the same HPLC retention times as AI-1 and AI-2 were isolated from PAO1 supernatants, where, from a comparison of the peak areas, the molar ratio of AI-1 to AI-2 was estimated as 15:1 (data not shown). MS analysis indicated that AI-1 and AI-2 had an m/z 172 (M + 1) and an m/z 200 (M + 1), respectively, by FAB-MS and MS–MS on molecular ion peaks, confirming that BHL and HHL are synthesized by *P. aeruginosa* (data not shown). The molecular ion of the compound provisionally identified from the HPLC analysis of PAO1 supernatants as OdDHL was at m/z 298 (M + 1) by FAB-MS, and by MS–MS, the 298 peak was identical in all respects to that of the synthetic OdDHL (data not shown).

Structure–Activity Relationships in the AI-Mediated Activation of VsmR. To gain insight into the structural requirements for the activation of VsmR by AHLs, a bioluminescent *E. coli*-based reporter system was constructed in which *vsmR* together with the *vsmI* promoter region of *P. aeruginosa* PAO1 was coupled to the *Ph. luminescens lux* structural operon. BHL and HHL activated this construct to a similar extent, with ≈1 μM being required for a half-maximal response (Fig. 5*A*). Extension of the N-acyl side chain to eight carbons to give OHL reduced activity to ≈50% that of BHL. The C_{12} acyl-chain analogue, dDHL, was inactive (Fig. 5*A*). To evaluate the importance of an oxygen substituent at the C-3 position of the N-acyl side chain, a second series of analogues was synthesized. OBHL, OHHL, and OOHL all exhibited similar but reduced activity when compared with BHL (Fig. 5*B*). OdDHL was more active than dDHL but was much less efficient at activating the reporter than each of the other analogues (Fig. 5*B*).

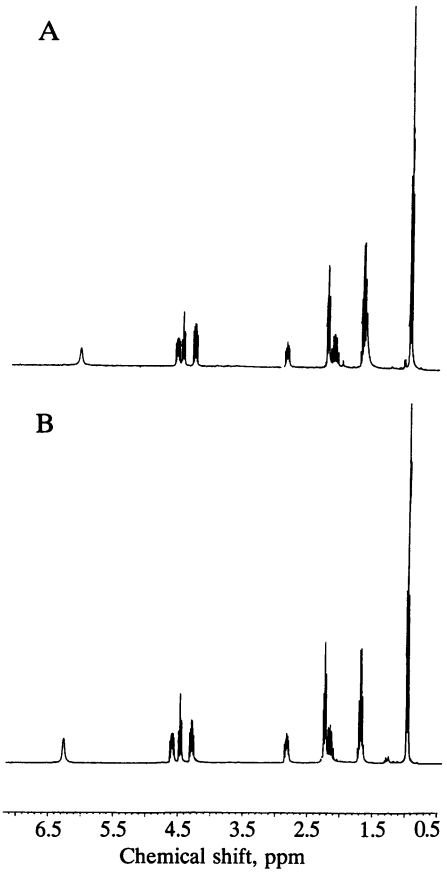

FIG. 3. High-resolution ^{1}H NMR spectra of AI-1 purified from spent culture supernatants of *E. coli* JM109(pMW47.1) (*A*) and synthetic BHL (*B*). Spectra were recorded in C^2HCl_3 with tetramethylsilane as the internal standard on a 400-MHz Bruker instrument. The assignment of the signals is as follows: δ 0.96 (3H, t, J = 7.4 Hz, CH_3), 1.68 (2H, sextet, J = 7.4 Hz, CH_3CH_2), 2.14 (1H, m 4α-H), 2.24 (2H, t, J = 7.1 Hz, CH_2CO), 2.85 (1H, m, 4β-H), 4.29 (1H, m, 5α-H), 4.47 (1H, td, J = 1.3 and 9 Hz, 5β-H), 4.56 (1H, m, 3-H), 6.06 (1H, br s, NH). The chemical shifts and integrated peak areas of the natural AI-1 and synthetic BHL were indistinguishable.

Biological Activity of BHL and HHL in *P. aeruginosa*. To determine whether BHL and HHL influence exoproduct production in a *P. aeruginosa* genetic background, we added either BHL or HHL to the PAN067 growth medium. Elastase synthesis was restored by either AI, although quantitatively BHL was more effective than HHL; at 100 μM, BHL restored elastase to around 58% of the wild-type PAO1 level (Fig. 6 *A* and *B*). Similar results (Fig. 6*C*) were obtained for chitinase (which degrades chitin, a β(1 → 4)-linked polymer of *N*-acetyl-β-D-glucosamine; ref. 27). BHL and HHL also restored cyanide synthesis in PAN067; at 100 μM, cyanide production was restored to ≈100% and ≈60% that of PAO1, respectively (data not shown).

DISCUSSION

Our data show that in *P. aeruginosa*, multiple AHLs regulate the production of virulence determinants and secondary me-

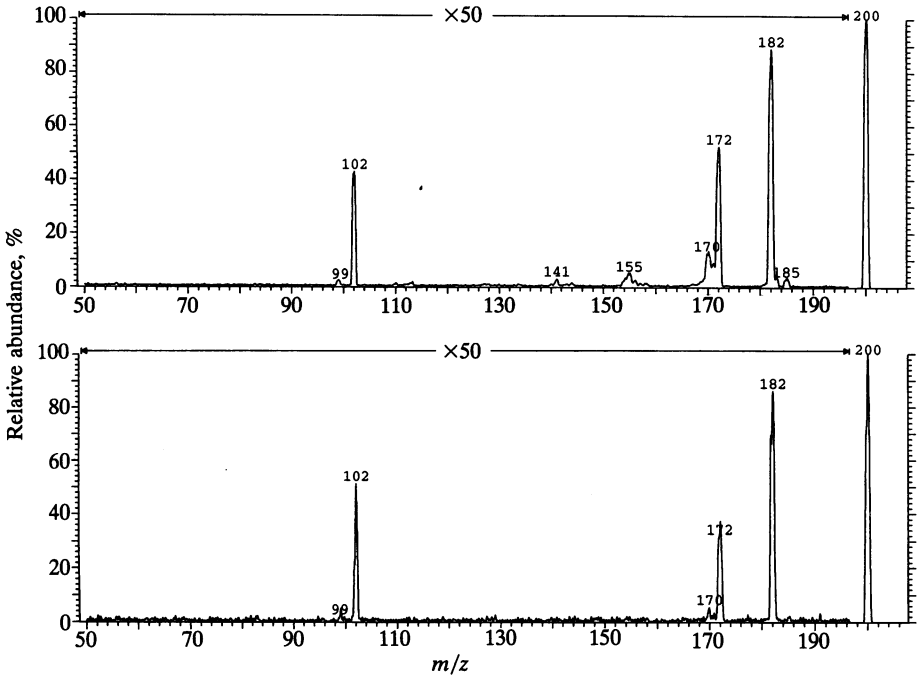

FIG. 4. Daughter-ion spectrum (MS–MS) of AI-2 (m/z 200 peak) purified from spent culture supernatants of *E. coli* JM109(pMW47.1) (*Upper*) is indistinguishable from that of synthetic HHL (*Lower*).

tabolites. *P. aeruginosa* produces AHLs which either have 3-oxo-substituted (OHHL and OdDHL) (2, 21) or unsubstituted (BHL and HHL) side chains. BHL and HHL add to the growing family of AHLs which includes OHHL in *Ph. fischeri* (28), *Er. carotovora* (2, 3), and *Y. enterocolitica* (19); OOHL in *A. tumefaciens* (27, 29), OHL in *Ph. fischeri* (30), and *N*-(3-hydroxybutanoyl)-L-homoserine lactone in *Vibrio harveyi* (36). Recently HHL has been identified in *Ph. fischeri* (30) and in *Y. enterocolitica* (19). Apart from *N*-(3-hydroxybutanoyl)-L-homoserine lactone, synthesis of these AHLs is directed by LuxI homologues, 10 of which have so far been cloned and sequenced (6, 8, 17). A striking feature of these putative AI synthases, which in general share only ≈30% identity at the amino acid level, is that they can direct the synthesis of the appropriate AHLs in both homologous and heterologous (*E. coli*) genetic backgrounds. In *P. aeruginosa*, LasI directs the synthesis of OdDHL and OHHL (21), whereas VsmI is responsible for BHL and HHL production. However, in both *Ph. fischeri* and *Y. enterocolitica*, the synthesis of both OHHL and HHL appears to depend on the same gene product—i.e., LuxI (30) and YenI (19), respectively. Why or how a single LuxI homologue is able to direct the synthesis of more than one AHL is not apparent.

The ability of HHL and BHL to restore exoproduct synthesis in a *P. aeruginosa* genetic background—i.e., in PAN067—resolves the apparent contradictions in the literature with regard to the regulation of elastase expression by OdDHL and OHHL (4, 21, 31). In particular, the inability of OHHL to activate a reporter system in *E. coli* containing *lasR* and a *lasB*::*lacZ* fusion (31) while being able to induce elastase production in PAN067 (4) can now be explained given the characterization of the *vsm* locus and the identification of BHL and HHL. Although OHHL cannot activate LasR (31), we

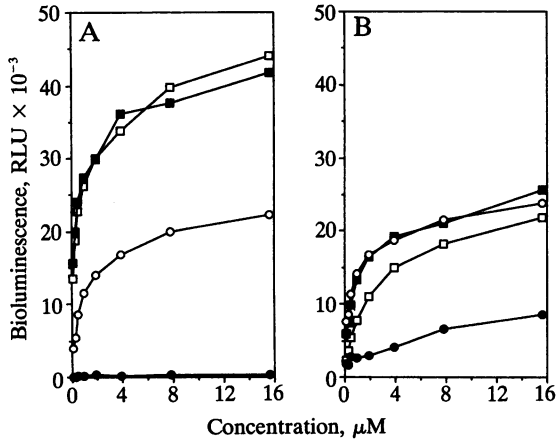

FIG. 5. Dose–response curves for the activity of BHL (□), HHL (■), OHL (○), and dDHL (●) (*A*) and their 3-oxo derivatives (*B*) in the *vsmR/vsmI*'::*lux* reporter assay. RLU, relative light units.

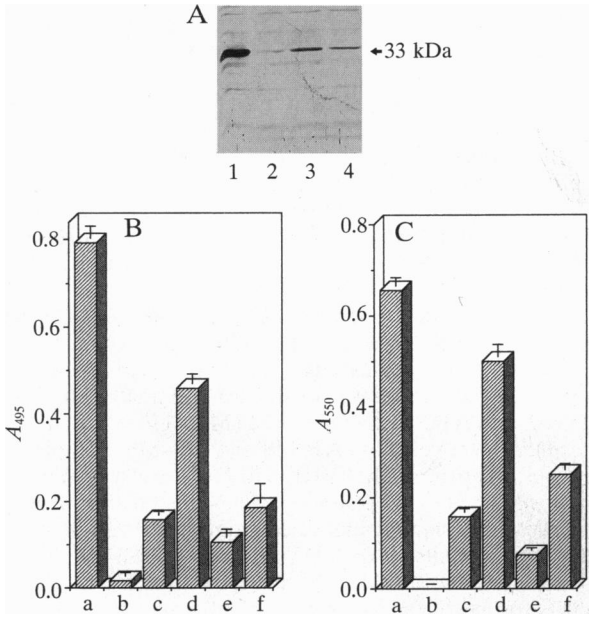

FIG. 6. (*A*) Immunodetection of elastase in culture supernatant of PAO1 (lane 1) or of PAN067 grown without added AHL (lane 2) or with BHL (lane 3) or HHL (lane 4) at 25 μg/ml. (*B* and *C*) Elastase assayed with elastin and Congo red (*B*) and chitinase production (*C*) in culture supernatant of PAO1 (bars a) or of PAN067 grown without added AHL (bars b), with BHL at 25 μM (bars c) or 100 μM (bars d), or with HHL at 25 μM (bars e) or 100 μM (bars f).

have used a *vsmR/vsmI'::lux* reporter assay to show that OHHL activates VsmR, albeit less efficiently than BHL. Conversely, the LasR AI OdDHL has little activity in this assay. Activation of VsmR is strongly influenced by the length of the N-acyl side chain and presence of an oxygen at the C-3 position; while introduction of 3-oxo into either BHL or HHL decrease activity by ≈50%, elongation of the N-acyl chain to C_{12} effectively abolishes activity. In contrast, the 3-oxo group and a C_{12} N-acyl side chain appear to be essential for optimal activation of LasR (32). For LuxR and the *Er. carotovora* LuxR homologue, CarR—which like VsmR depend on short-chain AHLs for activation—the C-3 substituent is critical. In *Er. carotovora*, carbapenem biosynthesis depends on the activation of CarR by OHHL (3, 10, 24); HHL and *N*-(3-hydroxyhexanoyl)-L-homoserine lactone exhibit <1% of the activity of OHHL (24). In *Ph. fischeri*, HHL has ≈10% the activity of OHHL (28). Thus, although the pattern of substrate specificity emerging for the LuxR homologues is high, it shows a certain degree of flexibility.

In PAN067, multiple phenotypes can be restored by the introduction of the *vsm* locus in multiple copies (17) or by the addition of exogenous BHL or HHL. Since BHL is the major compound produced and it is more active than HHL in restoring exoproduct synthesis, we presume that BHL is the natural ligand. However, on a molar basis, HHL is as effective as BHL in activating the *vsmR/vsmI'::lux* fusion. It is therefore possible that HHL regulates the expression of phenotypes other than those examined here. Further, to restore exoproduct synthesis to levels >50% of wild type required the addition of 100 μM BHL. It is not clear why such a high concentration is required since half-maximal activation of the *vsmR/vsmI'::lux* reporter requires only ≈1 μM BHL in an *E. coli* genetic background, but possible explanations are that (*i*) PAN067 is a chemically induced mutant (4) and may contain other mutations and (*ii*) there may be additional layers of regulation which are not overcome by the addition of a single exogenous AI. In *C. violaceum* (23) and *A. tumefaciens* (33), regulatory elements in addition to the corresponding LuxR homologue have been identified which modulate the response of the organism to AHLs.

P. aeruginosa PAO1 is the first bacterium in which two LuxR homologues (LasR and VsmR) and two LuxI homologues (LasI and VsmI) have been described alongside their cognate AHLs. The expression of certain phenotypes (e.g., elastase and alkaline protease) appears to depend on both quorum sensing systems (17, 34). This implies that the two systems may interactively regulate common target structural genes such as *lasB*. In PAO1, the upstream regions of both *vsmI* and *lasB* contain an almost identical *lux* box-like regulatory element which is probably the binding site for either or both LasR and VsmR (6, 17). It is therefore possible that VsmR and BHL/HHL activate expression of *lasR*, so that LasR in association with OdDHL activates *lasB*. Indeed, Pearson *et al.* (35) reported that OdDHL is unable to activate the *lasB* promoter in a *P. aeruginosa lasR* mutant when *lasR* is controlled by its own promoter. Instead, they observed that a second signal molecule, identified as BHL, was required. Further work is needed to elucidate the relationship between the various components of the *P. aeruginosa* quorum sensing circuitry. Such information ultimately may offer an approach for controlling *Pseudomonas* infections by attenuating virulence gene expression through the use of molecular antagonists which interfere with AHL-mediated signal transduction.

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